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Free-living amoebae (FLA) co-occurring with legionellae in industrial waters

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Abstract

Legionella pneumophila is known as the causative agent of Legionnaires' disease and free-living amoebae (FLA) can serve as vehicles for legionellae. The aim of this study was to screen industrial waters for the occurrence of FLA and their co-occurrence with legionellae. A total of 201 water samples, including 129 cooling waters and 72 process waters, and 30 cooling lubricants were included in the study. Treated waters were screened periodically, pre and post treatment. Altogether, 72.6% of the water samples were positive for FLA, acanthamoebae being most prevalent (in 23.9% of the samples) followed by *Vermamoeba vermiformis* (19.4%). Only one cooling lubricant was positive (*Acanthamoeba* genotype T4). *Legionella* spp. were detected in 34.8% of the water samples and in 15% in high concentrations (>1000 CFU/100 ml). Altogether, 81.4% of the *Legionella*-positive samples were positive for FLA by standard methods. By applying a highly sensitive nested PCR to a representative set of random samples it was revealed that *Legionella* spp. always co-occurred with *Acanthamoeba* spp. Although the addition of disinfectants did influence amoebal density and diversity, treated waters showed no difference concerning FLA in the interphases of disinfection. It appears that FLA can re-colonize treated waters within a short period of time.

Keywords: *Acanthamoeba*; Free-living amoebae; Industrial water; *Legionella*; PCR

Introduction

Free-living amoebae (FLA) are ubiquitous protozoa, that have been detected in various environmental and man-made habitats, including freshwater, brackish water, seawater, soil, air-conditioning units, drinking water treatment plants and even dental irrigation units (De Jonckheere 1991; De Jonckheere 2006; Michel et al. 1994; Michel and Just 1984; Stockman et al. 2011). In their natural environment, FLA are an essential part of microbial communities, they regulate bacterial populations by feeding on them, but they can also serve as hosts, vehicles and reservoirs of pathogenic and potentially

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pathogenic bacteria, including *Legionella* species (Greub and Raoult 2004; Michel et al. 1997; Rowbotham 1980). Particularly representatives of the genus *Acanthamoeba* are suitable hosts, because their cysts are extremely resistant against environmental stress like desiccation and changes in pH, osmolarity or temperature, they even survive chlorination or other disinfection methods (Kilvington and Price 1990). Representatives of the former genus "*Hartmannella*", recently shown to belong to different genera (Lahr et al. 2013; Smirnov et al. 2011), are also known to harbour legionellae. *Legionella pneumophila*, responsible for Legionnaires′ disease and Pontiac fever, is ubiquitous in natural and man-made freshwater environments and has evolved mechanisms to invade the amoebal host cell actively by inducing "coiling phagocytosis" and to evade being digested by inhibiting the fusion of phagosomes with lysosomes (Abu Kwaik et al. 1998; Albert-Weissenberger et al. 2007). After intracellular replication the legionellae are set free in vesicles or by lysing their host cell. When bacteria-filled aerosols are inhaled, the legionellae can infect alveolar macrophages. It has been shown, that growth within FLA, notably *Acanthamoeba*, not only protects legionellae from adverse environmental conditions, but amoebal passage also enhances virulence of *L. pneumophila* (Cirillo et al. 1994; Nora et al. 2009). Furthermore after intra-amoebal replication legionellae are less susceptible to biocides and disinfectants (Dupuy et al. 2011; Hwang et al. 2006). Cooling towers are a well-known source of human infections with *Legionella* spp. (Buse et al. 2012; Nguyen et al. 2006). However, in the past years, also outbreaks of legionellosis related to aerosol-spreading units in the timber and paper industry have been described (Nygård 2005; Nygård et al. 2008). The aim of our study was to evaluate the occurrence of FLA and their co-occurrence with *Legionella* spp. in process waters from the Austrian paper industry and compare these waters to water samples from cooling towers.

Material and Methods

Sample collection

In paper mills, water is used, cleaned and reused in many stages of the paper making process. Water samples were taken at paper machines and sewage plants. Sites with periodic disinfection measures were screened periodically, immediately before and after treatment and in the respective interphases between treatments. Altogether, 72 process water samples from the Austrian paper industry and 129 water samples from cooling towers were investigated. Additionally 30 cooling lubricants (composed of water and lipophilic components) were sampled. A staff member of each plant was requested to fill out a questionnaire on the sampling site. The water samples were collected in sterile plastic bottles, 3 L from each sampling site. Samples were processed within 24 hours and each water sample was mixed well before use.

Chemical and physical analyses

The water samples were analysed directly without any additional treatment. The cooling lubricants, 100 ml each, were centrifuged and the aqueous phase was investigated. Besides the pH, electrical conductivity (measure for total ion concentration), redox potential (indication of oxidizing agents) and concentrations of inorganic ions (ion chromatography) and of TOC (total organic carbon) were measured.

Screening for bacteria

Bacterial screening was performed by cultivation techniques according to standard methods. Water samples were analysed for *Legionella* spp. after concentration of 100 ml by filtration and centrifugation, respectively, cooling lubricants were directly used for analysis. Samples were analysed after heat and acid treatment as well as without treatment (according to the international standard ISO 11731:1998). Presumptive colonies were serologically identified by latex bead agglutination. *Pseudomonas aeruginosa* was evaluated in 10 ml sample volumes (EN 12780:2002/ISO 16266:2006) and total heterotrophic bacteria as colony counts in 1 ml sample volumes and 10-fold dilutions thereof after aerobic incubation at 36 °C and 22 °C (ISO 6222:1999).

Screening for FLA

For amoebal screening, 100 ml were drawn from each water sample and used for vacuum filtration through a cellulose acetate membrane (pore size 0.8 μm, area 12.5 cm^2, Sartorius, Germany). After filtration, 3 punches were taken of each filter and used for DNA-extraction. The rests of the filter membranes were placed onto NN (non-nutrient) agar plates coated with 100 μl of a 48 h old culture of *Escherichia coli* in brain heart infusion (BHI). Cooling lubricant samples (1 ml each) were centrifuged at 2000 × *g*/7 min and the pellet was resuspended in 200 μl of PBS, of which 100 μl were inoculated onto coated agar plates, the other aliquot was used for DNA isolation. All plates were sealed with Parafilm® and incubated at room temperature for one week. During that time they were examined daily for amoebic migration from the filters. First differentiation of the amoebae was accomplished by inverted phase contrast microscopy (Nikon TMS). FLA detected on the plates were cut out of the agar with a sterile scalpel and sub-cultured onto freshly bacteria-coated NNA plates. All isolates were cloned by successive subculturing and identified morphologically down to the genus and/or species level (in case of *Acanthamoeba* spp.: groups I-III) by inverted phase microscopy (Nikon TMS) and phase contrast microscopy (Nikon Eclipse E800) using the key of Page (Page 1991).

All *Acanthamoeba* isolates were subjected to genotyping as described below and the morphological identification of all *Naegleria* isolates and all isolates of representatives of the former genus *Hartmannella* (i.e. *Hartmannella cantabrigiensis*, *Nolandella abertawensis* and *Vermamoeba vermiformis*) was confirmed by sequencing a fragment of their respective 18S rDNA as described under "Additional PCR protocols". Generally, for molecular analysis, trophozoites were harvested from each clonal culture with sterile cotton swabs into a 1.5 ml tube filled with 0.9% sodium chloride (NaCl) for molecular analysis. The tubes were centrifuged for 10 min at 500 × *g*, the supernatant was discarded and the pellet was resuspended in 200 μl sterile phosphate-buffered saline (PBS) and freeze-thawed for cell rupture. Extraction of total genomic DNA was performed according to the "tissue protocol" of the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany). All PCRs were performed in 50 μl reactions, with DNA-free water as a negative control.

Genotyping of *Acanthamoeba* isolates

Acanthamoeba genotyping was performed by amplifying the 423–551 bp *Acanthamoeba*-specific amplimer ASA.S1 of the 18S rRNA-gene using the primers JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′ (Schroeder et al. 2001) and 1 μl and 3 μl whole cell DNA, respectively. Amplification conditions were: 15 min pre-heating at 95 °C, followed by 45 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and final extension for 7 min at 72 °C. A cloned ASA.S1 amplicon of a T4 genotype strain was used as a positive control. Amplified DNA was detected by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Amplicons were extracted from the gels with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and directly sequenced in both directions with the ABI PRISM® BigDye sequencing kit and an ABI PRISM 310® automated sequencer (PE Applied Biosystems, Langen, Germany). *Acanthamoeba* genotypes were determined with the model assumption of a <5% sequence dissimilarity within one genotype, as established previously (Gast et al. 1996).

Additional PCR protocols

As a proof of principle, 26 random samples from 17 Austrian paper mills were screened for FLA by PCR, namely for *Acanthamoeba* spp., *Naegleria* spp. and for representatives of the former genus *Hartmannella*. DNA was extracted from the filter punches using the "dried blood spot protocol" of the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany). DNA concentrations were measured using the NanoDrop® ND-1000 (220–750 nm spectrum, PEQLAB, Germany) and adjusted to 15–20 ng/μl. For detection of *Acanthamoeba*, two PCRs were employed, the standard JDP-PCR described above and a new highly sensitive nested PCR. The latter was established using the DNA extracted from filter samples of a dilution series from 10^5 cells/ml to 1 cell/ml of the reference strain *Acanthamoeba polyphaga* strain 4Cl, genotype T4 (ATCC PRA-107^{TM}) (Walochnik et al. 2000). The detection limit was below one cell. The first PCR step used the primers JDP1 and P3rev 5′-CTA AGG GCA TCA CAG ACC TG-3′ (Walochnik et al. 2004), amplifying an ∼800 bp fragment, under reaction conditions of 15 min at 95 °C, followed by 30 cycles at 95 °C for 1 min, 52 °C for 2 min, 72 °C for 3 min and final extension for 7 min at 72 °C. The second PCR step was performed with 3 μl of the first PCR product respectively, using the primer pair P2fw 5′-GAT CAG ATA CCG TCG TAG TC-3′ (Walochnik et al. 2004) and JDP2, amplifying an amplicon of about 180 bp. Reaction conditions were: 15 min at 95 °C, followed by 30 cycles at 95 °C for 1 min, 54 °C for 2 min, 72 °C for 3 min and final extension for 7 min at 72 °C. For detection of *Naegleria*, a sequence of 230 bp was amplified using the primers Nae3-For 5′-CAA ACA CCG TTA TGA CAG GG-3′ (Schild et al. 2007) and the universal eukaryotic primer binding to the 3′-end of the 18S rRNA-gene, NII 5′-AAA TGA TCC CTA CGC AGG TT-3′, under following reaction conditions: 15 min at 95 °C, followed by 30 cycles at 95 °C for 1 min, 52 °C for 2 min, 72 °C for 3 min and final extension for 7 min at 72 °C. This PCR was also used to confirm the morphological identification of all isolates, however, sequencing of the amplicon does not allow an identification below the genus level. For all former "*Hartmannella*" species, namely *H. cantabrigiensis*, *N. abertawensis* and *V. vermiformis* a new PCR was established, that covers all three species – as it was unclear which of these species are involved in bacterial survival. As this PCR would also detect closely related tubulinid genera as e.g. *Saccamoeba*, which are however rarely found in such water samples, all amplicons of the random samples were subjected to DNA sequencing. Moreover, this PCR was also used to further identify all isolates of representatives of the former genus *Hartmannella* obtained by culture. Primer design of HARTfor 5′-GGAGGGCAAGTCTGGTGCC-3′ and HARTrev 5′-GCCCGGAGAGTCATCCATG-3′ was based on an alignment of 18S rRNA gene sequences of all major amoebozoan groups obtained from the NCBI National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/) and including the reference strains *Hartmannella cantabrigiensis* (AY294147), *H. abertawensis* (=*Nolandella abertawensis*) (DQ190241) and *H. vermiformis* (=*Vermamoeba vermiformis*) (DQ084366, AF426157). The PCR results in an amplimer of approximately 520 bp length. Following reaction conditions were used: 15 min at 95 °C, followed by 30 cycles at 95 °C for 1 min, 58 °C for 2 min, 72 °C for 3 min and final extension for 7 min at 72 °C. The *H. cantabrigiensis* (AY294147) strain was used as a positive control. Amplified DNA was detected by gel electrophoresis and extracted from the gels and directly sequenced in both directions as described above. All obtained sequences were compared to

Table 1. Observed frequencies of occurrence of free-living amoebae (FLA) in water samples, retrieved from cooling towers (cooling water) and paper plants (process water).

Prevalence of FLA		Sample source		
		Cooling water	Process water	Total
FLA	Number	102	44	146
	Rate	79.1%	61.1%	72.6%
Acanthamoeba	Number	32	16	48
	Rate	24.8%	22.2%	23.9%
V. vermiformis	Number	23	16	39
	Rate	17.8%	22.2%	19.4%
Acanthamoeba co-occurring with *V. vermiformis*	Number	6	1	7
	Rate	4.7%	1.4%	3.5%
Others[a]	Number	42	20	62
	Rate	32.6%	27.8%	30.8%
Total	Number	129	72	201

[a] FLA other than *Acanthamoeba* and *V. vermiformis*, including *Echinamoeba*, *Vannella*, *Rhizamoeba*, Vahlkampfiidae (e.g. *Naegleria*), *Flamella* and unknown FLA.

sequences available in GenBank using NCBI Nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments for the genera *Acanthamoeba*, *Naegleria* and the representatives of the former genus *Hartmannella* respectively, were performed using ClustalX (Thompson et al. 1997). Sequence data were processed with the GeneDoc sequence editor (Nicholas et al. 1997).

Statistical analysis

The collected data were analysed with PASW Statistics software, version 18 (SPSS Inc., Chicago, USA), using crossing tables and chi-square test (asymptotic significance, 2-tailed). Significance was set at $p < 0.05$.

Results

As shown in Table 1, out of a total of 201 water samples, 146 (72.6%) were positive for FLA by culture. Water samples collected from cooling towers were to a significantly higher percentage positive for FLA with 79.1%, than process waters with 61.1% (Pearson chi-square, asymptotic significance, 2-tailed = 0.006; $p < 0.05$). *Acanthamoeba* spp., occurring in 23.9% of all investigated samples, were most prevalent followed by *Vermamoeba vermiformis*, detected in 19.4%, of all samples. The *Acanthamoeba* genotype found most frequently was by far genotype T4. Genotype T5 was found three times and one representative of each, genotype T9 and T11 was isolated. All representatives of the former genus "*Hartmannella*" that were found in this study belonged to the species *V. vermiformis*, showing >99% sequence identity to all other sequences of *V. vermiformis* (or *H. vermiformis*) available in GenBank in the sequenced fragment of the 18S rDNA. Other genera of FLA, such as *Echinamoeba*, *Flamella*, *Rhizamoeba*, *Vannella* and Vahlkamfiidae, and some unidentified amoebae were found in altogether 30.8% of the samples, from these 3 samples were positive for *Naegleria*. Absolute and relative frequencies are shown in Table 1. In seven out of 201 water samples *Acanthamoeba* spp. co-occurred with *V. vermiformis*. Interestingly, acanthamoebae and other FLA were detected more frequently in water samples from cooling towers, while *V. vermiformis* was found more frequently in process waters. 52 samples (25.9%) revealed at least two different genera of FLA, with highest amoebal diversity in cooling waters. Only one of the cooling lubricants was positive for FLA, namely for *Acanthamoeba* genotype T4. While concentrations of legionellae were generally higher with high redox values and lower with high TOC values, the occurrence of FLA did not correlate to water conditions.

Information on the use of biocides was provided for 174 out of the 201 water samples. Altogether 131 water samples had been treated regularly with heat and/or biocides, mainly sodium hypochlorite combined with sodium bromide or isothiazolinones, out of which 71.8% harboured FLA. It was shown that although immediately after disinfection less samples were positive for FLA and also amoebal diversity was smaller, there was no statistically significant difference between these waters in the interphases of disinfection and waters without any addition of disinfections. However, representatives of the Vahlkampfiidae were found, with two exceptions, only during phases without addition of disinfectants. Acanthamoebae and *V. vermiformis* did not show clear distribution patterns concerning water quality or time-point of disinfection.

Altogether, 34.8% (70/201) of the investigated water samples were positive for *Legionella* spp. and 19.9% (40/201)

Table 2. Comparison of standard methods and additional PCR methods for the detection of *Acanthamoeba*, *Naegleria*, and the representatives of the former genus *Hartmannella* (of which however, only *Vermamoeba vermiformis* was found) from a random sample of 26 process waters from paper plants.

Prevalence of amoebae		Process water	
		Standard methods[a]	Additional PCR[b]
Acanthamoeba	Number	6	23
	Rate	23.1%	88.5%
Naegleria	Number	0	1
	Rate	0.0%	3.8%
V. vermiformis	Number	6	6
	Rate	23.1%	23.1%
Acanthamoeba co-occuring with *V. vermiformis*	Number	0	5
	Rate	0.0%	19.2%
Total	Number	26	26

[a] Cultivation method and *Acanthamoeba*-PCR using primers JDP1/2.

[b] *Acanthamoeba*-nested PCR using primers JDP1/P3rev and P2fw/JDP2, *Naegleria*-PCR using Nae3For/NII primers and "*Hartmannella*"-PCR using HARTfor/rev primers in addition to standard methods.

were positive for *L. pneumophila*. By standard diagnostic methods *Legionella* co-occurred with FLA in 57 out of 70 (81.4%) *Legionella* spp.-positive samples, and this syntopic occurrence was statistically significant ($p = 0.049$). With 33.3% (19/57) a significantly high percentage of *Legionella*-positive samples also contained *Acanthamoeba* spp. In 28.1% (16/57) of the samples *V. vermiformis* was detected and one of the *Naegleria*-positive samples also contained legionellae, namely *L. pneumophila*. Among 70 *Legionella*-positive samples, 57.1% (40) were positive for *L. pneumophila* and amongst these, *L. pneumophila* co-occurred with *V. vermiformis* in 5 samples (12.5%). Almost twice as many samples showed *Acanthamoeba* with *L. pneumophila*, namely 27.5% (11). High concentrations of legionellae with more than 1000 CFU/100 ml were detected in 30 samples. With 80% (24/30) significantly more cooling waters than process waters (20%; 6/30), showed high *Legionella* concentrations, but in both cases approximately 80% of the samples with >1000 CFU/100 ml also contained FLA. *Pseudomonas* spp. were found in almost 50% of the samples and in some samples in >30,000 CFU/ml, particularly in samples taken during phosphate precipitation (period with no disinfection).

The results from the 26 random samples also screened for *Acanthamoeba* spp., *Naegleria* spp., and representatives of the former genus *Hartmannella* by PCR are shown in Table 2. While cultivation and standard PCR resulted in 6 (23.1%) *Acanthamoeba*-positive samples of these 26 random samples, the additional screening with the newly established nested PCR enabled the detection of *Acanthamoeba* spp. in 23 (88.5%) of the samples. All 26 random samples were negative for *Naegleria* spp. by cultivation, but one was positive by *Naegleria*-specific PCR. Four of the 26 samples were *Legionella*-positive and 2 of them were also positive for *L. pneumophila*. By using nested PCR, *Acanthamoeba* spp. were detected in all 4 *Legionella*-positive samples, whereas using standard methods, only one of these was positive for *Acanthamoeba*. Additionally to *Acanthamoeba*, one of these 4 *Legionella*-positive samples, one was also positive for *Naegleria* spp. and one for *V. vermiformis*.

Discussion

The occurrence of FLA in cooling towers and process waters from paper plants and their co-occurrence with legionellae in these waters was investigated for the first time. We demonstrate a wide distribution of FLA, including also potentially pathogenic taxa. Altogether, 72.6% of all water samples investigated were positive for FLA, with *Acanthamoeba* being most prevalent, followed by *V. vermiformis*. Water samples collected from cooling towers were to a significantly higher percentage positive for FLA than process waters, furthermore, cooling towers also showed higher amoebal diversity.

A general predominance of *Acanthamoeba* in water samples was recently shown in a study screening drinking and waste water treatment plants by culture and PCR in Spain (Magnet et al. 2013) and in that study genotype T4, being the most frequent genotype in our study, was the only genotype found. In a similar study screening different sites of a drinking water network in France and using culture and pyrosequencing "*Hartmannella*" (referring to *V. vermiformis*) was found to be by far the most prevalent "genus" (Delafont et al. 2013.). *V. vermiformis* was also very common in our study. *V. vermiformis* is the only known species within the newly established genus *Vermamoeba* (Smirnov et al.

2011) and all sequenced representatives of this species are very closely related showing <1% sequence dissimilarities in their 18S rRNA genes. The other representatives of the former genus *Hartmannella*, *H. cantabrigiensis* and *Nolandella abertawensis*, were not found in the current study, however, *Nolandella* is considered to be a mostly marine genus and *H. cantabrigiensis* altogether seems to be a very rare species (Lahr et al. 2013; Smirnov et al. 2011). Both genera, *Acanthamoeba* and *Vermamoeba* (in most studies referred to as *Hartmannella*), are known as important hosts and vehicles of legionellae (Thomas et al. 2008; Hsu et al. 2011). In accordance with the higher susceptibility to desiccation and detergents of *Naegleria*, particularly its cysts, compared to acanthamoebae (Marciano-Cabral 1988), only three samples were positive for *Naegleria* spp. Although representatives of the genus *Naegleria* have also been reported to harbour legionellae (Declerck et al. 2005; Thomas et al. 2008), these amoebae are most likely of minor importance as hosts for *Legionella* spp. in treated waters like industrial waters. In the current study the vahlkampfiids, to which also the genus *Naegleria* belongs, were the only FLA clearly susceptible to disinfection. Although the addition of disinfectants did influence amoebal density and diversity altogether, treated waters showed no difference concerning FLA in the interphases of disinfection. It appears that FLA are able to recolonize treated waters within a short period of time.

As was shown for FLA, also *Legionella* spp. were detected much more frequently in cooling towers (41.9%) than in process waters (22.2%). 18.8% of the cooling tower-samples showed high concentrations (>1000 CFU/100 ml) compared to 8.3% of the samples from paper plants. This was also proven by quantification of *L. pneumophila* by the CARD-FISH method (Kirschner et al. 2012). These findings comply with the assumption that cooling towers promote the formation of biofilms and therefore the growth of amoebae and their associated bacteria (Pagnier et al. 2009). Microorganisms in biofilms are more resistant against biocides, including free chlorine (Morton et al. 1998). In the current study, untreated samples were only slightly more positive for legionellae than treated samples, with 45.5% compared to 31.5% (not significant). This is most probably due to the fact that the currently applied disinfection protocols in the investigated plants, relying on regular heating and/or regular treatment with sodium hypochlorite/sodium bromide or isothiazolinones, did not significantly influence the occurrence of acanthamoebae and *V. vermiformis*. Both genera are known to be highly resistant against antimicrobial treatments and both are important vehicles for legionellae. It has been shown that *L. pneumophila* can survive for at least 6 months in a starvation medium in association with *A. castellanii* (Bouyer et al. 2007). Within acanthamoebae, *L. pneumophila* has been shown to survive 1024 p.p.m. of NaOCl, moreover non-culturable *L. pneumophila* may become resuscitated by passage through amoebae (Garcia et al. 2007).

The newly established *Acanthamoeba* nested PCR was significantly more sensitive than culture or conventional PCR. Thereby it was shown, that *Legionella* spp. indeed always co-occurred with *Acanthamoeba* spp. Thus, it can be assumed that standard methods are not always sensitive enough for industrial water samples. Compared to clinical samples, as e.g. cornea scrapings of patients suffering from *Acanthamoeba* keratitis, the amoeba density is much lower in water samples, and particularly low in industrial waters, moreover, the overall species diversity–complicating diagnostic PCRs–is much higher. The newly established nested PCR proved to be a specific and at the same time highly sensitive method for the detection of *Acanthamoeba* spp. in these special water samples.

Besides their role as vehicles for bacteria, members of the genus *Acanthamoeba* and possibly also *V. vermiformis* (referred to as *H. vermiformis*) are also facultative pathogens (Lorenzo-Morales et al. 2007; Moore et al. 1985; Martinez and Visvesvara 1997; Naginton et al. 1974). *Acanthamoeba* genotype T4, the predominant genotype in the current study, is the most important causative agent of *Acanthamoeba*-keratitis (AK), an infection that concerns particularly people wearing soft contact lenses (Risler et al. 2013), and as has been shown recently, the genotype most often associated with human disease (Maciver et al. 2013). Of the other genotypes found, genotypes T5 and T11, have also been described to cause keratitis (Iovieno et al. 2010; Lorenzo-Morales et al. 2011). Interestingly, T5 has been reported to be found most often in water samples (Maciver et al. 2013). Furthermore, several genotypes of *Acanthamoeba*, including particularly also T4, can cause granulomatous amebic encephalitis (GAE), a life-threatening infection of the brain occurring mainly in immunocompromised hosts as e.g. AIDS patients.

In conclusion, this is the first time that industrial waters were screened for the co-occurrence of amoebae and legionellae. We demonstrated that despite disinfection, both, potentially pathogenic amoebae and legionellae, are wide spread in cooling and – to a lesser extent – also in process waters. Fifteen percent of the water-samples showed high *Legionella* concentrations. By applying a highly sensitive nested PCR to a representative set of random samples it was revealed that all *Legionella*-positive samples were also positive for *Acanthamoeba*. Altogether, it can be stated that industrial waters are a possible source of infection with, both, *Acanthamoeba* spp. and *Legionella* spp., particularly for workers with an impaired health status.

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